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(54) BIOLOGICAL PRE-TREATMENT OF LIGNOCELLULOSE TO REMOVE LIGNIN

(71) We, GENERAL ELECTRIC COMPANY, a Corporation organized and existing under the laws of the State of New York, United States of America, of 1 River Road, Schenectady, 12305, State of New York, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to the biological pretreatment of lignocellulosic materials to degrade the lignin significantly.

The bulk of renewable organic matter on earth consists of lignocellulose. The cellulose component of this material is a linear polymer of glucose, and in a pure or relatively pure form it can be converted to a variety of useful products such as paper, meat, milk, sugar, ethanol and methane. Except in the form of cotton and some bacterial polymers, cellulose does not occur pure naturally but is present in the tissue of land plants complexed with lower molecular weight, alkali-soluble polysaccharides collectively termed hemicelluloses and with lignin, a high molecular weight three-dimensional random polymer of phenylpropane alcohols. Lignin protects cellulose from enzymatic hydrolysis to soluble sugars. The greater the lignin content of lignocellulose, the more resistant is its cellulose component to enzymatic attack.

Cellulose can be freed of lignin by physical means, e.g. fine grinding, and by chemical extraction at elevated temperatures, but both methods are expensive relative to the value of the final product.

A number of mold species have been shown to degrade lignin enzymatically, but their use has not been commercially attractive because they grow so slowly. Typically, two months or more are required to effect degradation of 50% of the lignin in a woody substrate. The lignin-degrading molds previously described have been mesophilic; that is, they grow best at temperatures of between 20—30°C.

The present invention relates to a thermotolerant mold which has been found to be a rapid degrader of lignin. Also, it has been found that the lignin is degraded appreciably only under damp—as opposed to submerged—conditions. In one aspect of the present process, lignocellulose can be delignified in order to make the cellulose available for subsequent fermentation.

The present process uses the thermotolerant mold, *Chrysosporium pruinosum*, which was obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. In the art *Chrysosporium pruinosum* is considered an equivalent of the thermotolerant molds *Phanerochaete chrysosporium* and *Sporotrichum pulverulentum*.

Briefly stated, the present process for degrading lignocellulose comprises providing a substrate composed of lignocellulose dampened with nutrient mineral solution at a pH from 4 to 5, said substrate being comprised of 10% by weight to 80% by weight of lignocellulosic solids based on the total amount of lignocellulosic solids and nutrient solution, inoculating said substrate with *Chrysosporium pruinosum*, maintaining the inoculated substrate at a temperature ranging from 20°C to 45°C to grow said *Chrysosporium pruinosum* which produces enzyme systems that degrade said lignocellulose, and elevating said temperature to a temperature ranging from 50°C to 70°C, at which elevated temperature said *Chrysosporium pruinosum* stops growing but at which degradation of said lignocellulose continues significantly.

Those skilled in the art will gain a further invention from the detailed description set forth below, considering in conjunction with the figures accompanying and forming a part of the specification, in which:

Figure 1 shows lignin and cellulose degradation by *Chrysosporium pruinosum* growing on a submerged substrate;

Figure 2 shows lignin and cellulose degradation by *Chrysosporium pruinosum* growing on a damp substrate; and

Figure 3 shows lignin and cellulose degradation by growing and temperature-shifted

(non-growing) cultures of *Chrysosporium pruinosum*.

In the present process any lignocellulosic material is useful. Representative of the lignocellulosic materials are wood, manure fiber, paper, straw, and agricultural wastes. Preferably, the lignocellulosic material is ground by any suitable means, preferably to a particulate size less than about 5 mm. to increase its surface area and thereby significantly or substantially increase the rate of degradation. Specifically, the more surface area provided by the lignocellulose, the quicker is the invasion process by the mold mycelium.

The particular nutrient mineral solution used is not critical except that it must have a pH of 4—5 to be operable for growing the mold. Specifically, the solution is largely inorganic comprised of a number of minerals in solution to provide the major nutrient ions such as sodium, potassium, phosphate, sulfate, magnesium and iron and usually includes an organic chelating agent to keep iron from precipitating and thiamine which is a necessary vitamin for *Chrysosporium pruinosum* mold growth and is included in the nutrient solution if it is not already present in the lignocellulosic substrate or if not present in sufficient amount to promote growth of the mold. The absolute concentrations of the nutrients in the solution are not critical as long as they are present in adequate amounts for the *Chrysosporium pruinosum* cells to grow but not so high as to inhibit growth. Standard bacteriological growing media are useful herein as nutrient mineral solution because they all contain the major ions necessary for mold growth, and the exact formulation may be modified in the standard manner depending on the composition of the particular lignocellulosic mass, i.e. the extent to which the nutrients are already contained in the substrate.

In carrying out the present process the lignocellulosic mass is dampened by the nutrient solution to produce the substrate on which the *Chrysosporium pruinosum* mold is grown. This can be done by a number of conventional techniques such as dropping or spraying the solution onto the lignocellulosic mass. When the mass is in particulate form, it can be admixed with the nutrient solution if desired. After addition of the solution to the lignocellulosic mass, a short period of time should be allowed for the solution to equilibrate, i.e. spread itself throughout the mass.

The lignocellulosic mass is contacted with an amount of nutrient solution to produce a damp substrate comprised of from about 10% by weight to about 80% by weight of lignocellulosic solids based on the total amount of lignocellulosic solids and amount of nutrient mineral solution. Amounts of lignocellulosic solids less than about 10% by weight result in no significant degradation of lignin whereas with amounts of solids higher than 80% by weight the mold does not grow. Satisfactory results are achieved at 20% by weight lignocellulosic solids concentration, i.e. 20 grams of lignocellulosic solids/80 grams of mineral solution +20 grams of lignocellulosic solids.

As used herein by a damp lignocellulosic substrate it is means a substrate comprised of 10% to 80% by weight of lignocellulosic solids based on the total amount of lignocellulosic solids and nutrient solution present. This is in contrast to a submerged substrate or condition wherein typically about 0.5% by weight of lignocellulosic solids is used based on the total amount of lignocellulosic solids and nutrient solution, i.e. 0.5 gram of lignocellulosic solids/99.5 grams of nutrient solution+0.5 gram of lignocellulosic solids.

The damp substrate may be inoculated with *Chrysosporium pruinosum* cells or with spores of the *Chrysosporium pruinosum* cells. The inoculation can be carried out by a number of techniques which allow as much contact as possible between the *Chrysosporium pruinosum* cells and the substrate. Preferably, the spores or *Chrysosporium pruinosum* cells are initially suspended in a liquid medium such as water usually at room temperature and the suspension dropped or sprayed onto the substrate.

The inoculated substrate is then incubated with sufficient aeration at a temperature ranging from 20°C to 45°C to grow the *Chrysosporium pruinosum* cells. The incubation can be carried out by a number of techniques which maintain the required growing temperature and also the required solids concentration such as a hot air incubator or by contacting the inoculated substrate with flowing hot air. preferably, for fastest growth of the mold, the growing temperature ranges from 38°C to 40°C.

While the mold is growing, i.e. incubating, it is consuming lignocellulose and producing enzyme systems indicated to be lignase for degradation of the lignin and cellulase for degradation of the cellulose.

Once the desired growth of mold is attained, the temperature is raised to an elevated temperature ranging from 50°C to 70°C at which elevated temperature it has been determined that mold growth stops but degradation of the lignocellulose continues. Preferably, the fastest degradation rates the elevated temperature ranges from 50°C to 60°C. Specifically, the present elevated temperature stops substrate utilization by the organism but allows the lignase and cellulase enzymes to continue to work. Elevated temperature conditions are maintained until a suitable amount of delignification is effected. Cellulose degradation will also be occurring during this treatment.

The period of time that the inoculated sub-

strate is incubated, i.e. the period that the mold is grown at a particular temperature, as well as the period of time at which the incubated substrate is maintained at a particular elevated temperature is determinable empirically and depends on the extent of degradation of the particular mass of lignocellulose desired. Specifically, after about three days of incubation the temperature can be elevated to a particular required elevated temperature and the incubated substrate held at such elevated temperature for a particular period of time, for example, three days, and then the extent of the degradation of the lignocellulosic mass determined in a conventional manner, generally by determining the amounts of lignin and cellulose remaining. This cycle can be repeated and graphs drawn to determine optimum periods of time and temperatures for a particular ligno-cellulosic material to yield the maximum amount of free cellulose or cellulase susceptible cellulose.

The resulting delignified or lignin-depleted material can then be used as a cellulose—or sugar-rich animal feed or fermentation substrate. The process will also produce soluble derivatives of lignin which can be useful as chemical feedstocks.

Besides being required for the process to work, the damp condition allows greater amounts of substrate to be treated per unit volume of culture relative to conventional submerged microbial processes.

The invention is further illustrated by the following experiments where materials and methods were as follows:

Organism

Chrysosporium pruinosum (Gilman et Abbott) Carmichael, ATCC 24782, was used in all experiments and was obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. This organism has recently been identified as the imperfect state of the fungus *Phanerochaete chrysosporium.*

Media

The nutrient mineral medium solution of the following composition was used: $(NH_4)_2SO_4$, 5.0 grams; KH_2PO_4, 6.04 grams; Na_2HPO_4, 0.85 grams; "trace elements" solution, 10 ml; distilled water added to 990 ml. The trace elements solution had the following composition: $MgSO_4 \cdot 7H_2O$, 5.0 g; $ZnSO_4 \cdot 7H_2O$, 0.2 g.; $FeSO_4 \cdot 7H_2O$, 0.5 g.; $MnSO_4 \cdot 4H_2O$, 0.5 g.; $CaCl_2$, 0.5 g.; versenol, 5.0 g.; distilled water to 250 ml. The pH of the medium was set at 5.0 before autoclaving by adding a few drops of concentrated HCl. An aliquote of a filter-sterilized solution of thiamine HCl (1 mg/ml) was added to the medium after autoclaving to give a final thiamine concentration of 1 μg/ml. Glucose-mineral-agar medium was prepared by adding 40 ml of a sterile 25% glucose solution to 960 ml of the hot sterile mineral medium described above containing 15 g of agar.

Preparation of the Lignocellulosic Substrate

Two hundred grams of dry manure were added to 2 liters of distilled water in a one gallon Waring blender and shredded at low speed for 15 seconds. The mixing was repeated three times with 15 second intervals between mixings. The suspension was transferred to a 30 cm. diam.×61 cm. high glass jar and diluted 1:1 with distilled water. This suspension was mixed with a propeller connected to a shaft and variable speed motor. The motor speed was set so that all material was suspended, and the suspension was stirred for 1.5 hours. The mixing speed was then reduced for 0.5 hr. to allow sand to settle while the lighter fiber particles remained suspended. With the motor running at reduced speed, the suspended particles were siphoned off and caught on a 20 mesh (0.238 mm openings) screen. This material was washed on the screen with distilled water: The stirring, settling, screening process was repeated three times, and the resulting fibers were dried at 65°C for three days. The dried fibers were ground in a meat grinder to redisperse the particles, and these were stored in glass jars.

Fiber was dried in a 65°C oven for at least two days prior to use until a constant weight was obtained. Since the material is somewhat hygroscopic in air, it was found convenient to place it in a covered petri dish on the surface of a hot plate while weighing samples. The hot plate surface was maintained at 100°C.

Dry particulate manure fiber prepared in this manner had an average size of 1—3 mm. in length and 0.5 mm. in width. It contained 14±1% by weight ash, 37±2% by weight reducing sugar (cellulose+hemicellulose), 37±1.5% by weight lignin and these are the amounts on which % by weight solubilized in Figures 1 and 2 were based.

Preparation of Inoculum

Cultures of *C. pruinosum* were grown at 38°C for 5 days on glucose-mineral agar plates. Ten ml of sterile distilled water was added to each plate, and the surface growth was gently suspended with a glass spreader. This suspension, which contained mainly spores and some fragments of vegetative mycelium was used as the inoculum for all cultures.

Conditions of Growth

Cultures were grown on submerged substrates in mineral medium solution in shake flasks and on damp substrates on the surface of mineral agar plates.

Submerged (shape flask) cultures contained 50 ml of mineral medium solution and 50 mg of dry, washed manure fiber in 250 ml erlenmeyer flasks. The flasks were autoclaved for

20 minutes and inoculated after cooling with 0.1 ml of the spore suspension described above. Cultures were incubated at 38°C at 80% relative humidity with rotory shaking at 230 rpm in a New Brunswick model G26 incubator-shaker.

Damp (agar surface) cultures were prepared in the following manner. Sterile Nuclepore membrane filters (5 μ pore size, 47 mm diameter), were placed, dull side down, on the surface of 25 mm×150 mm petri plates containing approximately 200 ml of mineral agar medium, composed of the nutrient mineral medium solution thickened with 1.2% by weight agar. One filter was used per plate. Two hundred mg of dry, sterile manure fiber was spread on the surface of each filter. The 200 mg fiber samples were dispensed from 16 mm×125 mm screw-capped Pyrex (Registered Trade Mark) tubes. This was equivalent to a 20% by weight fiber/20% by weight fiber+80% by weight nutrient mineral solution. Each pile of fiber was inoculated at the periphery with two drops (0.1 ml) of spore suspension prepared as described above. All cultures were incubated at 38°C. Relative humidity in the incubator was maintained at 80% using water-filled trays.

Analytical Methods

1. Measurement of residual organic and inorganic constituents of fiber and fiber cultures for Figures 1, 2 and 3.

a. Agar plate cultures (Damp Cultures). Filters containing fiber or fiber plus mycelium were removed from the agar plates, and the fiber-mycelial mats were scraped off the filters and washed with distilled water into tared crucibles. The crucibles were dried at 65°C to a constant weight and then ashed ovenight in an oven at 550°C. The organic fraction was defined as that material which vaporized at 550°C.

b. Shake flask cultures (Submerged Cultures).

The contents of a pair of shake flasks were 0.4 μ, 47 mm diameter Nuclepore filter to trap particulate materials. Residue remaining on the sides of the flasks was washed onto the filter with a minimal amount of distilled water. The filtered residue was transferred to tared crucibles and dried and ashed as described above.

2. Analysis for cellulose, hemicellulose and lignin for Figures 1, 2 and 3.

Samples of insoluble material were collected as described above except that they were transferred to 25 mm i.d.×50 mm high glass weighing vials and dried at 65°C. Dry samples of fiber or fiber plus mycelium were mixed with 10 ml of 72% sulfuric acid and allowed to stand for three hours with hourly mixing for a few seconds. These were then diluted to 50 ml with distilled water and allowed to stand overnight. Each sample was then vacuum-filtered through a tared 0.4 μ, 47 mm Nuclepore filter. Aliquotes of the filtrate were retained for carbohydrate (reducing sugar) assays. In this assay 0.1 ml of filtrate was mixed with 3.9 ml of a reagent containing 1.0% thiourea and 0.05% anthrone in 72% H_2SO_4. The mixture was heated to 95°C for 10 minutes, cooled to room temperature, and the optical density at 610 mμ was measured. Glucose was used as a standard. These assays were routinely performed using a Technicon model AA-1 autoanalyzer.

The residue on the filter was washed with distilled water until the filtrate pH reached five as measured with pH paper. The filter plus residue was dried overnight at 65°C and weighed. The filters and samples were then transferred to tared crucibles and ashed overnight at 550°C. Lignin was defined as the fraction of the sample (excluding the filter) which vaporized. The filters contained less than 0.5 mg ash.

For the data in Figure 3 inoculated damp fiber cultures on agar plates were prepared as described above. After 3 days one series of cultures was heated to a temperature of 55°C. and the resulting degradation of lignin and cellulose compared to that of a series of cultures which had been left at the growing temperature of 38°C.

Results

Figures 1, 2 and 3 illustrate the present invention. In Figures 1 and 2, % by weight solubilized is based on initial content in the fiber sample.

Figure 1 illustrates the degradation of manure fiber constituents by *Chrysosporium pruinosum* growing in submerged (shake flask) cultures. Each data point represents the combined contents of two shake flasks. Single points indicate that replicates give identical values. Dashed lines indicate uninoculated controls.

Figure 1 shows the loss of various fiber components with time in submerged (shake flask) cultures and that little or no lignin is solubilized, i.e. degraded, by *C. pruinosum* under submerged conditions relative to the uninoculated controls over the thirty-day period of active degradation. About 50% of the carbohydrate (cellulose and hemicellulose) fraction and 40% of the total organics are solubilized in this time period.

Figure 2 illustrates the degradation of manure fiber constituents by *Chrysosporium pruinosum* growing in damp fiber on the surface of mineral agar plates. Each data point represents the contents of one plate. All experiments were run in duplicate. Dashed lines indicate uninoculated controls.

Figure 2 shows the loss of various fiber components with time in damp fiber cultures on the surface of mineral agar plates and illus-

trates that extensive degradation of lignocellulose occurs preferentially on non-submerged substrates, i.e. damp substrates, and that a solids concentration of about 20% by weight allows rapid lignocellulose degradation. Specifically, fifty percent of the lignin, 80% of the carbohydrate (cellulose and hemicellulose) and 75% of the total organics are solubilized after 12 days of incubation after which degradation appears to stop. Less material is solubilized in the uninoculated controls under these conditions than in the shake flasks.

Table I shows lignin and cellulose degradation rates for *Polyporus versicolor*, a well-studied mesophilic lignin-degrading mold, and for *C. pruinosum* the present thermotolerant lignin-degrading mold as illustrated in Figure 2.

TABLE I

Lignocellulose Degradation by a Mesophilic Mold (Polyporus Versicolor) and a Thermotolerant Mold (Chrysosporium Pruinosum)

Organism	% Cellulose Degraded	Time	% Lignin Degraded	Time
P. versicolor[a]	76	245 days	50	168 days
C. pruinosum	80—90	12 days	50	12 days

[a] E. B. Cowling, Comparative Biochemistry of the Decay of Sweetgum Sapwood—by White-Rot and Brown-Rot Fungi. U.S.D.A. Tech. Bull. #1258, (1961).

Figure 3 shows that significant degradation of lignin and cellulose continues at a temperature of 55°C., i.e. a temperature at which the mold cannot grow.

The data indicate that under appropriate cultural conditions enough lignin can be removed biologically to make 85—90% of the cellulose susceptible to degradation by cellulase. The rate and yield data for cellulose degradation shown in Figure 2 are minimum values because the cell wall sugar residues contribute to the cellulose values obtained, and the cultures used were started from small spore inocula. Using a larger inoculum of cellulase and "lignase"-induced vegetative cells, i.e. the *C. pruinosum*, would presumably shorten the incubation period required to reach maximum cellulose degradation. Figure 3 shows that even though the cells were raised to temperatures at which they cannot grow (55°C) the enzymatic lignin and cellulose degradation proceeded in the absence of viable cells. From preliminary cellulose studies it was found that the optimum temperature of cellulase activity is above 55°C indicating that the present temperature shift during growth will turn off cell metabolism and permit continued saccharification.

Figure 2 shows that significant amounts of lignin can be degraded, and 80—90% of the substrate's cellulose content can be exposed to the action of cellulase enzymes. The manure fiber used in the experiments outlined in Figures 1—3 is rich in lignin relative to other natural organic materials. Cellulose digestability is inversely correlated with lignin content, suggesting that the present thermotolerant mold's performance on a resistant substrate such as manure fiber indicates equal or better digestive capacity on substrates containing less lignin such as wood. In fact, additional experiments have shown good lignin and cellulose digestion using newsprint as a growth substrate. Newsprint is derived largely from lignified wood fibers.

Our copending British Patent Application No. 35155/77 (Serial No. 1,560,021) claims a process for degrading lignocellulose which comprises providing a substrate composed of lignocellulose dampened with nutrient mineral solution at a pH from 4 to 5, said substrate being comprised of 10% by weight to 80% by weight of lignocellulosic solids based on the total amount of lignocellulosic solids and nutrient solution, inoculating said substrate with *Chrysosporium pruinosum*, and maintaining the inoculated substrate at a temperature ranging from 20°C to 45°C to grow said *Chrysosporium pruinosum* which produces enzyme systems that degrade said lignocellulose and allowing said mold to grow at said temperature until at least a significant amount of said lignocellulose is degraded.

WHAT WE CLAIM IS:—

1. A process for degrading lignocellulose which comprises providing a substrate composed of lignocellulose dampened with nutrient mineral solution at a pH from 4 to 5, said substrate being comprised of 10% by weight to 80% by weight of lignocellulosic solids based on the total amount of lignocellulosic solids and nutrient solution, inoculating said substrate with *Chrysosporium pruinosum*, maintaining the inoculated substrate at a temperature ranging from 20°C to 45°C to grow said *Chrysosporium pruinosum* which produces enzyme systems that degrade said lignocellulose, and elevating said temperature to a temperature ranging from 50°C to 70°C, at which elevated temperature said *Chrysosporium pruinosum* stops growing but at which degradation of said lignocellulose continues significantly.

2. A process according to claim 1 wherein

said substrate is inoculated with a liquid suspension of spores of said *Chrysosporium pruinosum*.

3. A process according to claim 1 wherein said substrate is comprised of about 20% by weight of lignocellulosic solids based on the total amount of said lignocellulosic solids and nutrient solution.

4. A process according to claim 1 wherein said inoculated substrate is maintained at a temperature of 38°C to 40°C to grow said *Chrysosporium pruinosum*.

5. A process according to claim 1 wherein said elevated temperature ranges from 50°C to 60°C.

6. A process according to Claim 1 and substantially as hereinbefore described with reference to Figs. 2 and 3 of the accompanying drawings.

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Sheet 1

FIG. 1

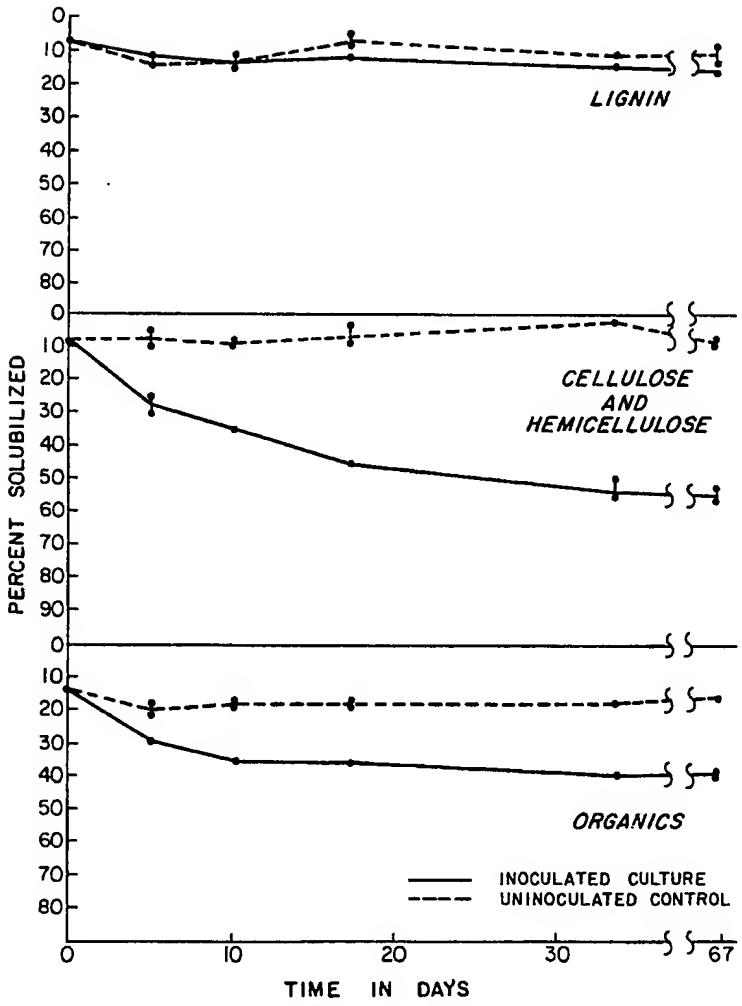
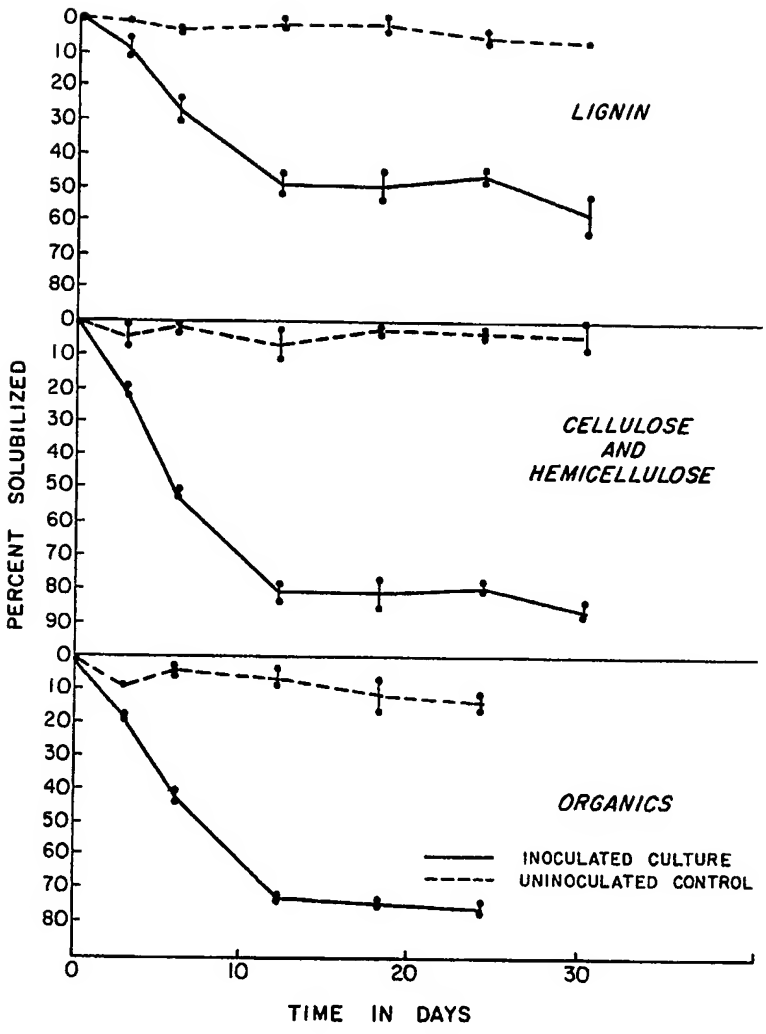


FIG. 2


PERCENT SOLUBILIZED
LIGNIN
CELLULOSE AND HEMICELLULOSE
ORGANICS
INOCULATED CULTURE
UNINOCULATED CONTROL
TIME IN DAYS
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1560022 COMPLETE SPECIFICATION
3 SHEETS *This drawing is a reproduction of the Original on a reduced scale*
Sheet 3

FIG. 3